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(54) Title: ELECTROPHORETIC QUANTITATION OF SPECIFIC BINDING COMPLEXES

(57) Abstract

Quantification of analytes is achieved by gel electrophoresis, where a plurality of samples may be analyzed simultaneously. By combining an analyte with its labeled complementary binding member and electrophoretically separating the complex from the unbound labeled complementary binding member, one can calculate the amount of analyte in the sample. A plurality of spaced apart samples may be placed in a single lane, so as to greatly expand the utility of a single gel plate.

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ELECTROPHORETIC QUANTITATION OF SPECIFIC BINDING COMPLEXES

INTRODUCTION

Technical Field

The field of this invention is electrophoretic identification of analytes.

Background

While electrophoresis is potentially an extraordinarily powerful tool to identify components of complex mixtures, identify specific analytes, provide for small pure samples of a wide variety of different biological materials, as well as providing other opportunities, for the most part the power of electrophoresis has not been realized. In part, this has been due to cumbersome methodology requiring substantial skill in setting up electrophoretic analysis, relatively long periods for performing the analysis, and lack of reproducibility from one analysis to another. Various procedures have been developed in order to try to alleviate some of these problems. Recently, U.S. Patent No. 5,104,512, issued describing a novel apparatus which affords substantially automatic monitoring of a gel electrophoresis. In addition, by affording control of various variables associated with the electrophoresis, reproducible results may be obtained at different times. Further, the apparatus affords real time monitoring of the course of the electrophoresis, so that one may control various parameters to change the parameters during the course of the electrophoresis.

In order to further supplement the power of gel electrophoresis, there is substantial interest in being able to devise new protocols which allow for more efficient utilization of the gel. Also, one is interested in relatively rapid simple methodologies for defining and quantitating amounts of analyte.

SUMMARY OF THE INVENTION

Gel electrophoresis protocols are provided for determining simultaneously one or a plurality of analytes in one or a plurality of samples simultaneously using a single gel plate. The methodology comprises combining the sample with a labeled complementary binding member in a pre-determined amount which allows for the formation of soluble complexes, removing insoluble material, and then applying the liquid medium at one of a plurality of wells present on the gel.

The gel may be characterized by having a plurality of lanes, with a plurality of sample wells or sample application sites in each lane, spaced apart a sufficient distance to preclude transport of a first sample past the next successive sample well. The difference between the amount of unbound labeled specific binding member in the gel band and the amount of the labeled specific binding member originally employed with the sample will indicate the amount of analyte present in the sample.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Protocols are provided for performing a plurality of gel electrophoretic determinations for the presence of one or more analytes. The method can be used to determine a multiplicity of samples, from the same or different source, on a single gel plate having a plurality of lanes, where each of the lanes has a plurality of application sites for applying the sample(s). The application sites are separated a sufficient distance, so as to allow for separation between a labeled specific binding member and a complex between the labeled specific binding member and the analyte.

The method comprises combining the sample with a predetermined amount of the labeled specific binding member to provide for soluble complexes. Any insoluble material may be separated and the liquid used as the sample. The sample(s) are then applied to the gel at the various application sites. The electrophoresis is then performed, whereby the labeled specific binding pair member and the complex of analyte and the labeled specific binding pair member are separated. One can then measure, one or both (assuming there is a single binding analyte) by means of the label, so as to quantitate the amount of analyte present in the sample.

The labeled specific binding pair member may be any compound, either ligand or receptor compound, which binds with high affinity, an affinity of at least about 10^7 mol^{-1}. The ligand may be a hapten, antigen, protein, sugar, lipid, nucleic acid, naturally occurring or synthetic small organic compound, combinations thereof, or the like. The receptor may be any compound which is complementary to the ligand, either naturally occurring or synthetic, usually being protein, but may also include nucleic acids. Common receptors include antibodies, surface membrane proteins, enzymes, lectins, etc., and one may also use nucleic acids where the nucleic acids may specifically hybridize to a complementary sequence.

The analyte will not be restricted by size for the most part, ranging from about 1 kD to 1,000 kD, usually not exceeding about 500 kD. Preferably, the analyte should not be more than about 10 fold different in molecular weight, more usually not more than about 5 fold different in molecular weight, from the complementary specific binding pair member.

Various labels may be used, including radioisotopes, fluorescers, enzymes, particles, and the like. Preferably, a fluorescent label is used. Fluorescent labels include fluorescein, Texas red, phycoerythirin, allophycocyanin, ethidium bromide, dimeric ethidium bromide, umbelliferone,

fluos, resos, cascade blue, luciferin yellow, TOTO, YOYO, bisethiduim, chelated rare earth metals, and the like. Usually, the fluorescer will absorb in the range of about 250 to 600 nm and fluoresce in the range of about 350 to 750 nm.

The gels can be employed with any convenient thickening agent, including agarose, acrylamide, gelatin, etc. In standard PAGE technology, gels commonly range between about 5-22.5% T (T = total amount of acrylamide or other gelling agent), mostly between about 7.5-15% T. Lower percentages may be employed with linear polyacrylamide. In agarose gel, electrophoresis, concentrations would generally range between about 0.2-2% T. Descriptions of forming gels for gel electrophoresis may be found in: The Practice of Quantitative Gel Electrophoresis, Chrambach, VCH Publishers.

The gel plate may be of any convenient size, generally ranging from about 5 to 40 cm in the direction of migration, and about 0.5 to 2 cm normal to the direction of migration, preferably about 0.5 to 1.5 cm. The gel thickness will generally range from about 0.5 to 1.5 mm. Alternatively, capillary electrophoresis may be carried out, where the capillaries are channeled which allows for application of a plurality of samples along the gel length.

In carrying out the assay, a buffer solution is employed, which may be any convenient buffer for preparing the reagents for combination with the sample. The sample may be any convenient physiological or non-physiological fluid, including blood derivatives, particularly plasma or serum, saliva, cerebrospinal fluid, *etc.*; fermentation and processing mixtures and streams; samples from natural sources, such as water, soil and air; food; etc. The buffer solution may employ any of the common buffers employed with gel electrophoresis, including Tris, glycine, Hepes, Mops, etc. Usually, the pH will be chosen to support the stabilization of complex formation between the specific binding pair members, generally the pH will be in the range of about 6 to 10. The buffer concentration will generally range from about 0.5 to 50 mM.

Where antibodies are used, either antisera or monoclonal antibodies may be employed and the particular concentration will be determined in accordance with optimizing the procedure emperically for determining the concentration of the antibody. Generally, the antibody will be present in an amount at least about 1.25 fold the highest concentration expected to be encountered in the sample and not more than about 5 fold, more usually not more than about 2 fold the highest concentration anticipated to be encountered in the sample. The ratio of antibody to analyte will be chosen in accordance with the Heidelberger curve (Heidelberger and Kandal (1929) J. Exp. Med. 50, 809; (1935) Ibid 62, 697; Heidelberger et al. (1946) Ibid 83, 303; and Heidelberger and Wolfram (1954) Fed. Proc. 13, 496). The analyte and its complementary binding member are usually combined in a volume ratio of about 0.1-1:1-0.1. The particular ratio will be determined by the relative concentration of the analyte in the sample, the potential for interfering agents in the sample, or the like. If desired, the sample may first be treated to remove interfering components, concentrate the analyte, change the nature of the analyte, for example, separating subunits or providing for subunits coming together, or the like. The sample and its complementary binding member are combined, usually conveniently at ambient temperature, or temperatures of from 4° to 40°C may be employed, and the mixture incubated for sufficient time for reaction to occur.

Desirably, controls will also be employed which allow for comparison of the sample results with the control results. The negative control may be water, buffer, the medium in which the analyte is present, a synthetic medium to mimic the analyte medium, or the like. The positive control will provide the analyte or comparable compound in the same or different medium in which the analyte is obtained.

After mixing the vials and incubating them, usually for at least about 1 min., more usually about 5 min. and not more than about 60 min., any sedimentation which occurs may be removed. The sedimentation may be removed conveniently by

centrifugation, although other techniques may be employed, such as filtration.

Aliquots or the entire sample may then be transferred to the gel at appropriate sites. The conditions which are employed for the electrophoresis may be optimized for particular analytes, usually being conventional conditions based on the nature of the receptor and the complex. Temperatures, voltages, and currents may be controlled as appropriate. Desirably, a separation will occur between the complex and the unbound labeled compound of at least about 5 mm, preferably at least about 10 mm, and more usually not more than about 25 mm, where there is a single complex. Where there is a plurality of complexes, the closest complex will generally be spaced at least about 5 mm from the unbound labeled complementary binding member.

After carrying out the electrophoresis, the bands may then be read and the amount of label in the bands quantitated. By knowing the amount of labeled complementary binding member, one may calculate the amount of uncomplexed labeled binding member or complexed binding member or both, so as to calculate the amount of analyte present in the sample. Thus, by calculating the amount of label in the faster moving zone, one can directly determine the amount of analyte in the sample.

An apparatus for carrying out the subject invention is extensively described in U.S. Patent No. 5,104,512, which patent is incorporated herein by reference. While this apparatus has many advantages for monitoring the electrophoresis in real time, other apparatuses will also find application which allow for appropriate detection of the movement of the bands.

The following examples are offered by way illustration and not by way limitation.

EXPERIMENTAL

The following is an exemplary test protocol, where the detection of human IgG is determined. The reagents comprise: buffer solution, Tris/glycine, 7.5 mM, pH 8.8;

anti-human IgG-antibody (LAB), either Texas red (TRITC) or Fluorescein (FITC) labeled; human IgG (AG) as AG-reference for positive control; agarose gel, 2% T in buffer solution pH 8.8.

Step 1: Immuno Reaction

All dilutions are carried out with buffer solution, pH 8.8. A 1 + 4 dilution of antibody (LAB-dil) is prepared and 100 µl of a human serum sample is combined with 100 µl of (LAB-dil) in an Eppendorf vial. The negative control replaces the sample with 100 µl of distilled water, while the positive control employs 100 µl of AG-reference (100% binding). The contents of the vials are mixed thoroughly without foaming in an incubator for 15 min. at 37°C. At the end of this time, the vials are centrifuged or 2 min. at 15,000 rpm to remove any precipitated material.

Step 2: Electrophoresis

Fifteen (15) µl of supernatants is employed to be applied to the sample sites of the gel. The gel electrophoretic conditions are as follows: 5°C, voltage limit: 500 V; current limit 35 mA; sample entry time 100 sec.; application position: anodic; 8 positions/application, 25 mm distance between samples; sample entry field strength: 5.0 V/cm, constant voltage; separation field strength: 22.5 V/cm, constant voltage. Using the previously indicated apparatus, the gels are scanned twice over time intervals of 6 min. A separation distance between the unbound antibody-zone and immuno complex of about 20 mM is achieved.

The subject methodology provides for numerous advantages. No staining or fixation steps are required and evaluation is performed in real time. One need only evaluate the fluorescent emitting zones, thus ensuring that only antibody (LAB) and antigen-antibody complexes (AGAB-CX) are evaluated. One need only determine the amount of unbound antibody, the fastest moving zone, in the present example the most cathodic zone. One can also determine the AGAB-CX-zone, particularly where a plurality of complexes are determined, so as to quantitate each of the complexes. Total antigen concentration (AG) can be calculated by

decrease of free LAB concentration against the LAB concentration of the negative control.

The concentration of insoluble immuno complexes is obtained by the difference between the sum of LAB plus AGAB-CX-concentrations obtained for the sample and the LAB-concentration in the negative control. The positive control uses a reference for the maximum for the AGAB-CX-concentration obtainable in accordance with the protocol. Samples having AGAB-CX-concentrations equal to the positive control require a repeat test with a higher dilution of the sample. The concentration range for the test evaluation may cover three to six orders of magnitude without a requirement for additional dilution steps prior to the immuno reaction.

It is evident from the above results, that a simple rapid test is obtainable where a plurality of samples may be simultaneously analyzed for the presence of one or a plurality of analytes in one or a plurality of samples. With an 8 lane gel, of 125 mM length, one can provide for 4 samples per lane, resulting in 32 determinations. Thus, a large number of samples may be simultaneously monitored. The method can use standard equipment in a novel and efficient manner to substantially expand the information that can be obtained in a single electrophoresis.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for quantitating an analyte in a sample by gel electrophoresis, said method comprising:

combining said sample with a predetermined amount of a labeled complementary binding member to provide soluble complexes of said analyte and said complementary binding member in a reaction medium;

applying said reaction medium to a gel and electrophoresing said reaction medium to separate said complementary binding member from said soluble complex into separate bands in said gel; and

determining the amount of said labeled conjugate in at least one of said bands as a measure of the amount of analyte in said sample.

2. A method according to Claim 1, wherein said complementary binding member is an antibody and said analyte is an antigen.

3. A method according to Claim 1, wherein said labeled complementary member is a fluorescent labeled complementary member.

4. A method according to Claim 3, wherein said fluorescent labeled complementary member is a fluorescein labeled complementary member.

5. A method according to Claim 1, wherein said sample is placed at a plurality of sites in a single lane, wherein said sites are at least about 25mm apart.

6. A method according to Claim 1, wherein said gel is an agarose gel.

7. A method according to Claim 1, wherein said gel has a plurality of lanes and said sample is placed at a plurality of sites in each lane, each of said sites being at least about 25mm apart.

8. A method for quantitating an antigenic analyte in a sample by gel electrophoresis, said method comprising:

combining said sample with a predetermined amount of a fluorescent labeled antibody complementary to said analyte to provide soluble complexes of said analyte and said fluorescent labeled antibody in a reaction medium;

applying said reaction medium to an agarose gel and electrophoresing said reaction medium to separate said antibody from said soluble complex into separate bands in said gel; and

determining the amount of said labeled antibody in at least one of said bands as a measure of the amount of analyte in said sample.

9. A method according to Claim 8, wherein said sample is applied to said gel at a plurality of sites in a lane, said sites being at least about 25mm apart.

10. A method according to Claim 8, wherein said antibody is a monoclonal antibody.

11. A method according to Claim 8, including the additional step of applying a control sample at a site on said gel, where said control sample has a known amount of analyte.

INTERNATIONAL SEARCH REPORT

I. national application No.
PCT/US93/09701

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C25B 7/00; G01N 33/557
US CL :204/182.8; 436/517
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 204/182.8; 436/517

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS
electrophoresis, quantitat?, analyte, bind?, soluble, complementary

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US, A, 5,137,609 (Manian et al.) 11 August 1992, col. 2, lines 14-22 and 34-36; col.4, lines 12-13; col.7, lines 22-23 and 55-58.	1-11 ------- 1-11
A	US, A, 5,055,415 (IMAI ET AL.) 08 October 1991.	

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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